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Purification of the *E.coli ogt* gene product to homogeneity and its rate of action on O^6-methylguanine, O^6-ethylguanine and O^4-methylthymine in dodecadeoxyribnucleotides

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ABSTRACT

The *E.coli* gene *ogt* encodes the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (O^6-AlkG ATase). The protein coding region of the gene was cloned into a multicopy expression vector to obtain high yields of the enzyme ($\sim$ 0.2% of total protein) which was purified to apparent homogeneity by affinity, molecular exclusion and reverse-phase chromatography. Good correlation was found between the determined and predicted amino acid compositions. The ability of the purified protein to act on O^6-methylguanine (O^6-MeG), O^6-ethylguanine (O^6-EtG) and O^4-methylthymine (O^4-MeT) in self-complementary dodecadeoxyribonucleotides was compared to that of 19 kDa fragment of the related *ada*-protein. With both proteins the rate order was O^6-MeG > O^6-EtG > O^4-MeT, however, the *ogt* protein was found to repair O^6-MeG, O^6-EtG and O^4-MeT, 1.1, 173 and 84 times, respectively, faster than the *ada* protein.

INTRODUCTION

The reaction of alkylating agents with DNA results in the formation of a number of base and phosphate modifications (1,2). Some of these have been shown to be toxic and mutagenic in bacteria and mammalian cells (3,4) which contain repair enzymes capable of correcting certain types of damage (1,3−5).

The repair of the lesion O^6-MeG was first demonstrated in *E.coli* following administration of *N*-methyl-N-nitro-N-nitrosoguanidine (MNNG) (6). Under such conditions repair is most likely to be mediated predominantly by the O^6-AlkG-ATase that is the product of the *ogt* gene (7). This 19 kDa protein transfers the methyl group from the O^6-position of guanine to a cysteine residue within the protein itself (7,8) a process which is stoicheiometric and autoinactivating.

Exposure of *E.coli* to low doses of MNNG results in an increase in their resistance to the toxic and mutagenic effects of a subsequent higher dose of MNNG, a phenomenon known as the adaptive response (9,10). This response is controlled by the *ada* gene (11) the product of which was later shown to be a 39 kDa protein that contained 2 ATase functions (8,12,13). One of these repairs O^6-AlkG and O^4-AlkT (15) and the other repairs the S- stereoisomer of alkylphosphotriesters (AlkP) (16). This ATase is very susceptible to proteolytic cleavage at amino acid residue 178 and gives rise to two subfragments of 19 and 20 kDa. The latter of these is responsible for the repair of AlkP and, in its alkylated form the 39 kDa protein has been shown to be a positive regulator of the adaptive response. This involves its binding to a region known as the *ada* box within the *ada* promoter and the subsequent upregulation and transcription of the gene (17).

The 19 kDa protein encoded by *ogt* does not repair AlkP (7,8) however the amino acid sequence demonstrates 29% homology to the C-terminal domain of the *ada* protein, the

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region responsible for the repair of O^6-AlkG (7). Analysis of the *ogt* promoter does not reveal any homology to the *ada* box sequences suggesting that *ogt* would not be upregulated during the adaptive response (7) and no evidence of upregulation has been found (Potter *et al*, unpublished results).

A number of experiments in which the characteristics of the *ogt* ATase are being compared to those of the *ada* ATases are in hand. In the present report we describe the purification of the *ogt* encoded O^6-AlkG-ATase to apparent homogeneity from extracts of bacteria harbouring *ogt* in a multicopy expression vector. The amino acid composition of the homogeneous protein has been determined and the protein has been used to measure its rate of action, in comparison with the *ada* encoded O^6-AlkG-ATase, on self-complementary synthetic oligonucleotides containing O^6-MeG, O^6-EtG or O^4-MeT.

MATERIALS AND METHODS

Bacteria and Plasmids

E.coli JM83 (18) harbouring the plasmid pO61M was used throughout this study. pO61M consists of a 1304 bp insert isolated from pO61SP1 (7) (see Figure 1) containing the complete promoter region and protein coding sequence of the *ogt* gene inserted in the unique *Sma*I site of pUC9 (19). pO62SX (20) contains 793 bp of the 3′ region of the protein coding sequence of the *ada* gene inserted into the multiple cloning site of pUC9 and encodes protein that only repairs O^6-AlkG. pO62HSR (Potter *et al*, unpublished results) contains all of the *ada* gene except for 157 bp of the 3′ end which encodes the active site for the repair of O^6-AlkG and O^4-AlkT.

Alkyltransferase Assay

Extracts of *E.coli* harbouring various plasmids, fractions collected during chromatography or pooled activity peaks were assayed for ATase activity as described elsewhere (7). Assays for AlkP ATase and O^6-AlkG-ATase activities individually involved use of modified [^{3}H]-methylnitrosourea treated DNA substrates from which a) O^6-MeG (and O^4-MeT) were removed by prior incubation with the O^6-AlkG ATase fragment of the *ada* gene encoded by the plasmid pO62SX (20) or b) AlkP were removed by incubation with the AlkP ATase fragment of the *ada* gene encoded by the plasmid pO62HSR. The preparation and verification of these substrates will be described more completely elsewhere.

Polyacrylamide Gel Electrophoresis (PAGE)

Protein extracts were analysed using an SDS discontinuous buffer system (20) adapted by Bury (22) and visualised with Coomassie Blue or by silver-staining (23).

Purification of the ogt Gene Product

Eight litres of *E.coli* JM83 harbouring pO61M were grown to stationary phase in LB medium and centrifuged to obtain approximately 15 g of cells. These were disrupted by sonication (three pulses of 2 minutes at 30 μ peak to peak with cooling on ice) in 100 ml of buffer I (50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol DTT pH 8.3). Phenylmethylsulphonyl fluoride was added to 0.5 mM immediately after the last sonication. Following centrifugation at 30,000 g for 10 minutes, the sample was applied to a double-stranded DNA-cellulose column (15 cm$\times$2.5 cm) equilibrated in buffer I. After washing extensively with the same buffer, proteins were eluted into 4 ml fractions with buffer I containing 0.1, 0.25 or 0.5 M NaCl. Monitoring of the eluant was performed at 280 nm. Fractions containing the ATase were pooled and concentrated to a final volume of 1.2 ml using an Amicon ultrafiltration system employing a YM10 membrane. The sample was then applied to a Sephacryl S200HR column (85$\times$1.5 cm i.d.) and proteins eluted

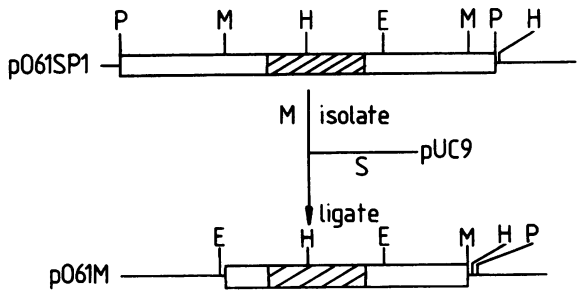

Figure 1. The plasmid pO61M was constructed by restriction enzyme digestion of pO61SP1 with *MnlI*, isolating the 1304 bp fragment containing the *ogt* gene and ligating this into *SmaI* digested pUC9. Resultant clones were analysed by digestion with *Eco*RI or *Hind*III. E, *Eco*RI; H, *Hind*III; M, *MnlI*; P, *PstI*; S, *SmaI*.

using buffer I. Fifty fractions (4 ml) were collected and following PAGE and ATase assay, the protein was located in 2–3 fractions. These were then further purified on a Vydac C4 reverse phase column (Technicol). Typically a linear gradient of 10–60% acetonitrile in 0.05% trifluoroacetic acid was run over 40 minutes and the eluate monitored at 214 nm. Fractions were then analysed by PAGE and silver staining.

Total Amino Acid Analysis

Approximately 5 μg of HPLC-purified ATase was analysed in triplicate by a ABI 420 analyser. Asparagine and aspartic acid and glutamine and glutamic acid are not distinguished by this procedure and hence the sum of these amino acids is shown in the Results.

O^6-Alkylguanine DNA Alkytransferases

a) ogt ATase. Homogeneous O^6-AlkG ATase (16 pmol/μl) was prepared as described and stored in aliquots in 50 mM Tris-HCl, 1 mM EDTA, 3 mM DTT pH 8.3 at −20°C.

b) ada Atase. Purified preparations of the *ada* encoded 19 kDa (fraction V, 5 pmol/μl, 23) O^6-AlkG ATase were generous gifts from Dr P Karran and Dr T Lindahl and were stored in 30 mM KH_2PO_4, 300 mM NaCl, 3 mM EDTA, 10 mM DTT, 50% glycerol pH 7.5.

Oligodeoxynucleotide Substrates

Self-complementary dodecadeoxynucleotides of the general type CGCalkGAGCTXGCG and CGCXAGCTalkTGCG, (where X is A,G,C or T), were synthesised by Dr B F L Li using the phosphotriester method in solution (24,25) and were purified by reverse phase HPLC on phenyl 4μ reverse phase radial compression cartridges (Waters) as previously described (26).

To determine the quality and quantity of each oligonucleotide, a small aliquot of each was subject to base analysis composition. The samples were digested to nucleosides by 30 min incubation at 37°C in 130 μl 60 mM Tris-HCl, 6 mM $MgCl_2$ pH 8.5 containing 14 μg phosphodiesterase and 5 μg alkaline phosphatase. Two 60 μl samples were loaded onto the radial compression C18 cartridge and eluted at 2 ml/min with a gradient of buffers A (50 mM KH_2PO_4 pH 4.5) and B (50 mM KH_2PO_4, 25% acetonitrile pH 4.5). Peaks were integrated with a Gilson 620 data module and the areas in the unmodified bases compared with those obtained from a standard mixture of each of the four nucleosides.

5'-($\beta^{32}P$)-labelling of Oligonucleotides

Oligonucleotides (0.36 A_{260} units) were ^{32}P labelled at 37°C in 70 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM spermidine pH 7.6, 10–20 units of T4 polynucleotide

kinase (Amersham) and 50−100 pmol of γ-^{32}P-ATP (3000 Ci/mmole, Amersham) in a volume of 50−100 μl. After 30 minutes incubation, 750 nmols unlabelled ATP was added and the reaction allowed to continue for a further 30 minutes.

The ^{32}P labelled oligonucleotides were purified by HPLC as described above.

Repair of Alkylated Dodecanucleotides by E.coli Alkyltransferases

The reaction between the oligonucleotide and the ATase was performed at 37°C in a reaction mixture containing 50 mM Tris-HCl, 10 mM DTT, 1 mM EDTA, 200 μg/ml BSA pH 7.6. Reaction kinetics were followed by quenching the reaction with 0.1 A_{260} units of the non-radioactive, phosphorylated, alkylated oligonucleotide. This was also used as a marker for the HPLC analysis. Quenched samples were loaded directly onto a Nova-pak 4 C18 or phenyl radial compression cartridge eluted at 3 ml/min with buffer C and a 1% per minute increasing gradient of Buffer D (0.33 M KH_2PO_4, 33% acetonitile pH 6.3).

In a second order reaction,

$$kt = \frac{1}{B_o - A_o} \cdot ln \frac{(B_o - x).A_o}{(A_o - x).B_o}$$

where k is the rate constant in M^{-1} sec^{-1}; B_o and A_o are the concentration of reactants at time = 0; and $B_o - x$ and $A_o - x$ are the concentration of reactants at time = t. The curved line that represents the progress of repair can be plotted linearly if

$$ln \frac{(B_o - x).A_o}{(A_o - x).B_o}$$

is plotted as a function of time

The rate constant (k) can be calculated since the slope of the line is equivalent to $k(B_o - A_o)$.

RESULTS

Overexpression of ogt Protein

Using modified substrates it was found that in various *E.coli* strains *O*^{6}-AlkG ATase activity is around 8 times higher than AlkP ATase activity (e.g see Table 1 and unpublished results). A number of different sublcones of the original pO61 (7,8) were produced in attempts to optimise *ogt* expression. The most effective combination for maximal expression was pO61M in JM83 in extracts of which *O*^{6}-AlkG ATase activity was at least 170 times higher than in the host: AlkP ATase expression was essentially unaffected by this high

Table 1 *O*^{6}-Alkylguanine and alkylphosphotriester DNA alkyltransferase activities in extracts of *E.coli* strain JM83

Extract	MNNG[1] treatment	Alkyltransferase activity (fmoles/mg protein) *O*^{6}-AlkG	AlkP
JM83	−	300	36
JM83	+	5040	6300
JM83/pO61M	−	50700	80

[1] − See text for experimental details

Table 2 Stages of Purification of the *ogt* Alkyltransferase

Fraction	Total Protein mg	mg recovered	Alkyltransferase[1] % of total protein	Units[2]	Specific activity Units/mg
Crude extract	2120	3.7	0.17	194740	91.9
DNA cellulose	2.09	0.92	44	48420	23170
Sephacryl S-200HR	0.23	0.22	98	12105	52630

[1] – Figures based on amount of 'active' ATase present in fraction
[2] – 1 unit = 1 pmole

level of *ogt* expression (Table 1). This contrasts with the concomitant increase in both O^6-AlkG and AlkP ATase activities following exposure of JM83 to MNNG (1 μg/ml) for 90 mins (Table 1). We were thus confident that if proteolysis of the endogenous *ada* protein occurred in or during the preparation of extracts of JM83 harbouring pO61M the contribution of the *ada* 19 kDa subfragment would be negligible i.e. maximally around 0.1–0.2% of the total O^6-AlkG ATase activity (Table 1).

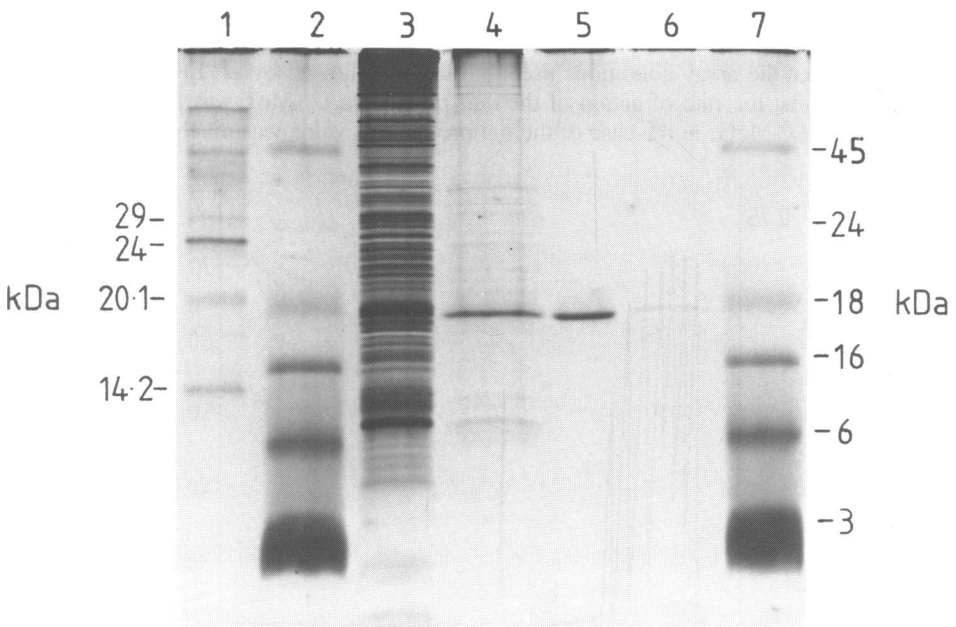

Figure 2. SDS-PAGE analysis of the purification of the *ogt* ATase. Samples were electrophoresed in an 18% resolving gel with a 3% stacking gel and visualised by silver staining. Lane 1, Sigma SDS-6 molecular weight markers (66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa, 14.2 kDa). Lanes 2 and 7, Prestained BRL low molecular weight markers (reported apparent molecular weights 45 kDa, 24 kDa, 18 kDa, 16 kDa, 6 kDa, 3 kDa). Lane 3, Crude bacterial extracts. Lane 4, Sample following DNA-cellulose chromatography; Lane 5, Sample following Sephacryl S-200HR chromatography. Lane 6, Sample following reverse phase HPLC.

Purification of the ogt Protein

E.coli JM83 harbouring the plasmid pO61M express greater than 50 pmol of ATase per mg of total protein (Table 2) and as a consequence this combination was used as the starting material for the purification of the *ogt* protein. Table 2 shows the results of the various stages of purification.

The ATase was essentially pure following Sephacryl S-200HR chromatography representing greater than 98% of total protein as shown by PAGE and silver staining (Figure 2). However, prior to amino acid composition analysis, any residual impurities were removed by reverse phase HPLC (Figure 3). Extensive loss of activity occurred during this procedure, presumably due to the denaturing effects of the organic solvents, hence the ATase specific activity for this stage of the purification is not shown. Activity of the ATase could be more effectively retained by collecting the eluted fractions into tubes containing an equal volume of 0.1 M Tris-HCl, 50% glycerol, pH 11.

Approximately 200 μg of homogeneous ATase were obtained by this purification procedure representing a 590 fold purification.

Amino Acid Composition

Table 3 shows the expected and the calculated results for the *ogt* gene product alongside those for the O^6-AlkG ATase subfragment of the *ada* protein. The expected figures were determined from the predicted protein coding sequence of the ATases (7,13).

Repair of Alkylated Bases in Oligodeoxyribonucleotides

The rate constants for the repair of various alkylated bases by the 19 kDa *ada* protein and the 19 kDa *ogt* protein are shown in Table 4. In both cases O^6-MeG is the preferred substrate under the assay conditions used and this is followed by O^6-EtG and O^4-MeT. However, whilst the rate of action of the *ada* protein on O^4-MeT was less than 0.01% of its rate on O^6-MeG, in the case of the *ogt* protein this value was much higher (0.7%).

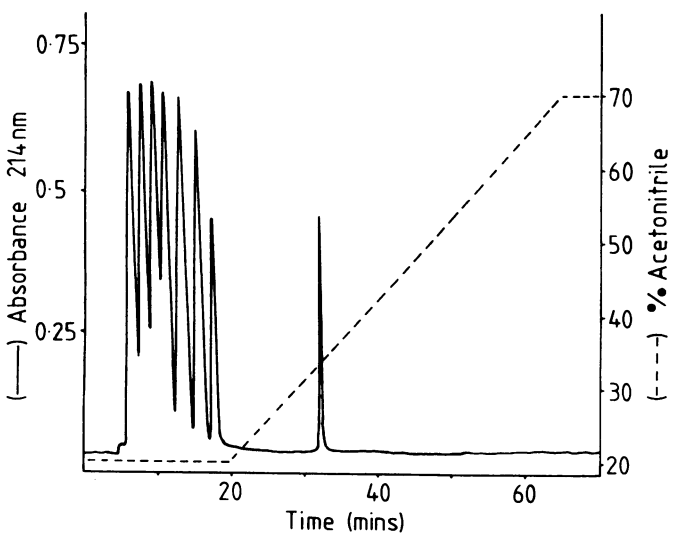

Figure 3. A_{214} Elution profile of reverse phase HPLC purification of the ATase. Multiple load peaks can be seen prior to the start of the gradient of acetonitrile. The pure *ogt* protein elutes at approximately 33 minutes (see text for details).

Table 3 Amino Acid Composition Analysis of the *ogt* Alkyltransferase

Amino Acid	*ogt* Observed[1]	*ogt* Expected	*ada* O^6-AlkG ATase[2]
Ala	12.1	12	24
Arg	15.5	15	16
Asn + Asp	10.7	11	15
Cys	ND	3	6
Cln + Gln	19.6	20	24
Gly	17.6	19	12
His	4.5	4	3
Iso	7.5	10	9
Leu	16.1	20	19
Lys	5.5	5	4
Met	4.3	4	2
Phe	3.4	3	4
Ser	6.1	6	8
Thr	10.1	10	6
Trp	ND	4	2
Tyr	7.5	7	3
Val	8.0	10	12
Predicted M.wt (Da)	–	19165	19311

ND – Not determined
[1] – The data was 'fitted' to a molecular weight of 18090 Da. This corresponds to the deduced molecular weight of the ATase minus those of the cysteine and tryptophan residues that were not determined by the analyser.
[2] – From reference 13

The corresponding values for O^6-EtG were 0.1% for *ada* and 15.5% for *ogt*. Consequently, whilst the *ogt* and *ada* proteins show similar rates of repair of O^6-MeG the *ogt* protein repairs O^4-MeT and O^6-EtG around 84 and 173 times faster respectively than the *ada* protein (Table 4).

Repair of Methylated DNA

Extracts of JM83 harbouring pO61M or pO62SX were purified by DNA cellulose chromatography (see above). Increasing amount of these samples from protein-limiting to substrate limiting were used on a standard ATase assay except that incubation was prolonged to 2h at 37°C. As found previously (7) the amounts of radioactivity transferred to protein under substrate limiting conditions were 8–12% higher for the *ada* O^6-AlkG ATase than the *ogt* ATase (Figure 4).

DISCUSSION

In order to facilitate the purification of the *ogt*-ATase we used a number of pO61 subclones and vector host combinations and the optimum combination was pO61M in JM83. We

Table 4 Rate Constants for the Repair of dodecadeoxyribonucleotides Containing Alkylated Bases

Substrate	*ada* O^6-AlkG ATase	*ogt* Protein	*ogt*/*ada*
O^6-MeG:C	260,000[1]	290,000	1.1
O^6-EtG:C	260 (0.1)[2]	45,000 (15.5)	173
O^4-MeT:A	25 (0.01)	2,100 (0.7)	84

[1] Rate constants $\times 10^2 M^{-1}$ sec^{-1}
[2] Figures in parenthesis are %, with rate for O^6-MeG:C oligonucleotide as 100%

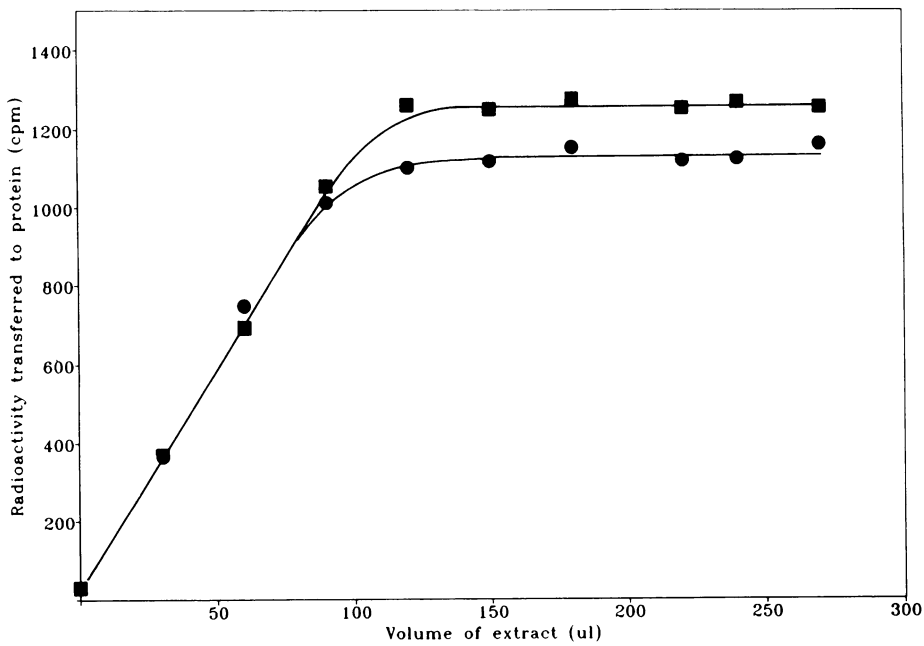

Figure 4. Alkyltransferase activity in extracts of *E.coli* JM83 harbouring pO61M (○ – ○) or pO62SX (■ – ■) following purification by DNA cellulose chromatography. See text for details.

calculated that the synthesis of *ogt* protein amounted to 0.1–0.3% of the total protein. Since JM83 is wild-type with respect to *ada* it was considered prudent to examine whether the overexpression of *ogt* ATase resulted in upregulation of endogenous *ada* gene expression. In comparison with the 175-fold increase in *ada* gene expression induced by adaptive treatment with MNNG (Table 1) little evidence was found for such upregulation and assuming that the *ogt* ATase and the 19 kDa *ada* protein subfragment would copurify, a maximum of 0.2% of the final product would be the *ada* ATase.

Purification of the *ogt* ATase was monitored by ATase assay and PAGE with silver staining. DNA cellulose chromatography followed by molecular exclusion chromatography produced an essentially pure product which retained most of its enzymic activity. Samples of this material were subjected to reverse-phase HPLC and this further demonstrated the purity of the material and provided samples for amino acid analysis.

With the possible exception of leucine, the molar ratios of the amino acids were very close to those predicted from the nucleotide sequence of *ogt* (7). The predicted molecular weight is 19165 Da, very close to that of the O^6-AlkG ATase subfragment of the *ada* protein (19311 Da). As reported previously (7) the *ogt* and *ada* O^6-AlkG ATases show 50% homology over a 30 amino acid residue region and 29% overall. This homology is reflected in the similarity in the amino acid compositions of the two proteins, the exceptions being cysteine and alanine which are twice as abundant in the *ada* protein and tyrosine, tryptophan and methionine which are twice as abundant in the *ogt* protein. The extent to which these differences result in differences in the shape of the molecules and

their substrate specificities may be clarified when the results of X-ray crystallography analyses are available.

The *ogt* and *ada* O^6-AlkG ATases acted at similar rates on oligonucleotides containing O^6-MeG and although the rate of alkyl group transfer from oligonucleotides containing O^6-EtG or O^4-MeT was in both cases lower than that for O^6-MeG, the *ogt* protein was considerably more efficient than the *ada* O^6-AlkG ATase. The repair of O^6-EtG confirms our previous conclusions about the *ogt*-protein (8). The *ada* ATase has been reported to act more slowly on O^6-EtG in DNA (27) or O^4-MeT in homopolymers (15) and these results confirm our present findings. The magnitude of the differences in rates between O^6-MeG, O^6-EtG and O^4-MeT between these and our present results are possibly due to differences in the nucleotide context. Although this might affect the rate of action of the enzymes it would be expected that all potential substrate lesions would eventually be repaired. Our earlier suggestion that the *ogt* protein might not be capable of repairing O^4-MeT in DNA (28) was based on the amount of radioactive methyl groups transferred to protein under substrate limiting condi-tions (7): this was and has remained (Figure 4) consistently lower than for the *ada* O^6-AlkG ATase and it appeared that the difference might correspond to the amount of O^4-MeT in the substrate DNA. However, the present results clearly demonstrate that the *ogt* ATase is able to act on O^4-MeT. One possible explanation for this discrepancy is that within some nucleotide sequences O^4-MeT or O^6-MeG are resistant to attack or are repaired very slowly. Further examination of this possibility is in progress.

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